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应用^{32}P 标记物检测 glnA 表达载体及转基因水稻愈伤组织中 NPT Ⅱ 酶活性

CONSTRUCTION OF EXPRESSION VECTOR CONTAINING glnA GENE AND DETECTION OF NPT Ⅱ ACTIVITY IN THE TRANSGENIC RICE CALLI USING ^{32}P-LABELLED COMPOUND

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摘　要

应用 PCR 技术扩增了编码巴西固氮螺菌 *Azospirillum brasilense SP7* 谷酰胺合成酶（GS）的基因 glnA. 扩增的 1.4 kb DNA 片段克隆于 Bluescript-SK 载体的 EcoRV 位点。序列测定结合限制性内切酶图谱分析，证实了携有 BamHI 位点的 1.4 kb DNA 片段确为巴西固氮螺菌 *SP7* 的 glnA 基因。该基因与 pCo24 的 Bgl Ⅱ 位点连接转化，筛选出以 CaMV35S 为启动子的 glnA 表达载体 pGSC35. 利用 α-^{32}P-dATP 标记探针进行菌落原位杂交筛选阳性克隆，经三次连接转化后，又构建了另一个带水稻肌动蛋白 1 启动子的 glnA 表达载体 pAGNB92. 由水稻 T986 悬浮细胞系分离出原生质体。然后，以 PEG 法和电激法，用均含有 NPT Ⅱ 基因的 glnA 表达载体 pGSC35 和 pAGNB92 转化水稻原生质体。转化后的原生质体于含有 G418 抗生素的培养基上进行筛选。以 γ-^{32}P-ATP 和卡那霉素为底物，经斑点杂交检测，37% 的 G418 抗性愈伤组织显示出 NPT Ⅱ 酶活性。

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CONSTRUCTION OF EXPRESSION VECTOR CONTAINING glnA GENE AND DETECTION OF NPT Ⅱ ACTIVITY IN THE TRANSGENIC RICE CALLI USING ^{32}P-LABELLED COMPOUND

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ABSTRACT

The glnA gene encoding glutamine synthetase (GS) was amplified from *Azospirillum brasilense Sp7* by PCR technique. The amplified 1.4 kb DNA fragment was cloned at the EcoRV site of Bluescript-SK. Both sequencing and restriction digestion data showed that the 1.4 kb DNA fragment flanked with BamHI site at each end was really the glnA gene of *A. brasilense Sp7*. The glnA gene was ligated with Bgl Ⅱ site of pCo24. As a result, an expression vector pGSC35 with CaMV35S promoter was obtained. Using colony *in situ* hybridization with α-^{32}P-dATP labelled probes to screen the positive clones, another glnA gene expression vector pAGNB92 with rice actin 1 promoter was constructed after three rounds of ligation and transformation. Protoplasts isolated from rice cell suspension line cv. T986 were transformed with glnA expression vectors pGSC35 and pAGNB92 containing neomycin phosphotransferase Ⅱ (NPT Ⅱ) gene by using PEG fusion and electroporation. Transformed microcalli were selected on media containing G418 disulfate salt. NPT Ⅱ activity was detected in 37% of G418 resistant calli by using dot blot hybridization with γ-^{32}P-ATP and kanamycin as substrate.

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INTRODUCTION

Rice is one of the most important crop in developing countries. Nearly half the world's population depends on rice as the major sources of nutrition calories. To feed the ever-increasing population of the developing region, the world's annual rice production must increase from 460 million tons to 560 million tons by year 2000[1]. Nitrogen is the key input required for rice production. Over past 2 decades, farmers have become increasingly dependent on chemical fertilizers as N source. At the same time the current recognition that increased industrial consumption of energy produced by burning fossil-fuel has led to world wide increase in atmospheric CO_2 and global warming. Reduction of energy consumption and increasing costs are becoming increasingly important. In this context, the biological nitrogen fixation-derived N assumes importance on the one hand. On the other hand, production of the transgenic rice which could grow normally on nitrogen poor soil is also a perspective ones.

Glutamine synthetase (GS, E.C.6.3.1.2.) is a key enzyme in ammonia assimilation either in plants or in gram-negative microorganisms including diazotrophs. It plays an important role in the efficient utilization of nitrogen sources and nitrogen metabolism in organisms. Peter reported previously that glnA gene encoding GS transgenic tobacco could overproduce GS and grow normally in N-poor soil[2]. In recent years, the transgenic rice and transformation of rice protoplasts have been made considerable progress[3,4]. The foreign gene expression with CaMV 35S promoter[5], maize Adh1 promoter[6] and rice actin 1 promoter[7] has been reported in cereal system.

In this paper the construction of glnA gene expression vectors with CaMV35S and rice actin 1 promoters and transformation of rice protoplasts as well as the detection of NPT II reporter gene flanked in expression vectors using ^{32}P-labelled compound have been described.

Abreviations used in this paper as follows:
B1, BamHI; R1, EcoRI; H3, HindIII; P1, PstI;
S1, SalI; Sm1, SmaI; K1, KpnI; X1, XbaI.
pBS, Bluescript-sk; P35s, CaMV35S promoter;
Sp, Spectinomycin; Kan, Kanamycin;
Act1-pro, rice Actin 1 promoter.
pGSC35, a recombinant plasmid with reverse insertion of glnA in pCo24.
G418, Geneticin disulfate salt, G418.

1 MATERIALS AND METHODS

1.1 Materials

1.1.1 Strains and Plasmids

glnA gene from *Azospirillum brasilense Sp7* and *E. coli DH5-a* were used as competent cell.

Plasmids: pAB444 was gifted by Dr. Elmerich[8]. pCOR 113 were from Prof. Ray Wu[9]. Bluescript-SK, pCo24 and pBIN 19 were from National Lab of Protein Engineering and Plant Genetic Engineering, Peking University.

1.1.2 Restriction Enzymes and Chemicals

All restriction enzymes, T_4 DNA ligase and nick-translation kit were purchased from Biolabs and Boehringer Mannheim Co. Nitrocellulose filter was from SABC. P_{81} phosphorus filter was purchased from Whatman Co. PEG-6000 and 8000 were from Sigma Co. α-^{32}P-dCTP and γ-^{32}P-ATP were from Amersham Co. and Beijing Furei Co. Agarose type VI was from Sigma Co. Cellulase "Onozuka" RS and Pectolyase Y-23 were purchased from Yakult Honsha Co. and Pharmaceutical Co. respectively.

1.1.3 Rice Cultivar Was T986 from China National Rice Research Institute.

1.2 Methods

1.2.1 Isolation of GlnA Gene from *A. brasilense Sp7*

(1) PCR amplification of glnA gene

glnA gene located in the 10 kb EcoRI-Hind III fragment of pAB444 which was used as template for PCR amplification of glnA gene. The isolation and purification procedure were described by Sambrook et al[10]. Primers used for glnA PCR were designed as follows according to the glnA sequence of *A. brasilense Sp 7*[11]:

Primer 1 : 5' AA GGATCC ATG TCC GAC ATC AGC AAG GTC 3'

Primer 2 : 5' CC GGATCC TCA GAC CGA GTA GTA CAT CTT 3'

(2) Cloning and sequencing of glnA gene

The amplified DNA fragment was blunted with Klenow enzyme and then cloned into Bluescript-SK vector. The ligation, transformation and digestion were carried out according to the method of Sambrook et al[10]. Double stranded DNA sequencing reactions were performed by using dideoxynucleotide chain termination method according to the protocol of T_7 polymerase kit (Pharmacia).

1.2.2 Construction of Expression Vectors Containing glnA Gene

(1) Construction of glnA expression vector with CaMV35S promoter

A 1.4 kb BamHI fragment (i. e. glnA gene) was excised from a clone containing glnA gene and then cloned at the Bgl Ⅱ site of pCo24. This recombinant plasmid is a glnA gene expression vector with CaMV35S promoter.

(2) Construction of glnA gene expression vector with rice actin 1 promoter

The protocol for this construction was showed in Fig. 5 including three rounds of ligation, transformation and twice colony in situ hybridizations to screen the positive clones.

1.2.3 Isolation and Culture of Rice Protoplasts

The procedure for cailus induction and maintenance, cell suspension cultures, isolation and culture of protoplasts were carried out by the method of Thompson et al[12]. Protoplasts were isolated from established cell suspension culture after 3～5 days subculture.

1.2.4 Transformation of Rice Protoplasts

Protoplasts were transformed with these two expression vectors as mentioned above by using PEG and/or electroporation methods. MaMg medium was used in PEG-mediated transformation with the final concentration of 19% PEG on the basis of the protocol provided by Zhang et al[13]. Electroporation was performed using the procedure described by Toriyama et al. [14] with slight modifications.

1.2.5 Selection of Transformants

The G418 antibiotic was added into the KR liquid medium when the transformed cell was at the third division stage in darkness at the concentration of 20 μg/ml. The G418^r microcalli were transferred into N_{6-1} differentiation medium containing 25 μg/ml of G418 for further selection.

1.2.6 Detection of NPT Ⅱ Activity in Transgenic Rice Calli

G418^r calli were picked up after 28 days selection and divided into two equal parts. Half of them was transferred to fresh LS medium to proliferate for 20 days. The proliferated calli were used to analyze NPT Ⅱ activity. NPT Ⅱ dot autoradiography was carried out by the method of Wang et al[15]. The other half parts of G418^r calli were transferred to N_6 differentiation and regeneration media to regenerate green plantlets for subsequent analysis such as Southern blot etc.

2 RESULTS

2.1 Amplification and Identification of glnA from pAB444

The electrophoretogram of PCR products after 35 cycles was shown in Fig. 1. The 1.4 kb fragment was recovered. To identify the PCR product, the recovered 1.4 kb fragment was cloned at the EcoRV site of Bluescriptt-sk. The clones with insertion were cut with EcoRI-Hind Ⅲ double digestion and BamHI single digestion. According to the known sequence of glnA from *A. brasilense Sp* 7[11], the recombinant plasmid (named pGSJ1) containing 1.4 kb BamHI fragment were further digested by SalI, PstI, SmaI respectively. The results of these restriction enzyme digestion were shown in Fig. 2, which correspond to the restriction sites of glnA from *A. brasilense Sp7*. Sequencing data for the two ends of the inserted fragment presented further evidence (autoradiogram not shown). The sequenced length reaches 213 bp for 5' end and 222 bp for 3' end, and no nucleotide mutation has been found. All these evidences indicate that the 1.4 kb BamHI fragment is undoubtedly the glnA gene of *A. brasilense Sp7*.

2.2 Construction of glnA Expression Vectors

2.2.1 Construction of Expression Vector pGCS35 with CaMV 35S Promoter

The glnA expression vector pGSC35 with CaMV 35S promoter was shown in Fig. 3, Fig. 4 indicated the results of restriction digestion and PCR amplification of pGSC35. The orientation of glnA gene in pGSC35 was determined by SalI digestion in comparison with Sal I site in pCo24. Two recombinant plasmids in either orientation were selected. As a result, plasmid as shown in lane A. D. was a CaMV 35S promoter directed glnA expression vector which was named pGSC35.

2.2.2 Construction of Expression Vector pAGNB92 with Rice Actin 1 Promoter

Rice actin 1 promoter was located in plasmid pCOR113[9]. However, there was neither terminal signal downstream the actin 1 promoter nor selectable marker gene in pCOR113. To add these two functional elements into this plasmid, three rounds of ligation and transformation including twice colony *in situ* hybridization had to be carried out. All these protocols were shown in Fig. 5.

Nos terminal signal (Nos-ter) with about 300 bp in length was isolated from pCo24 and blunted with klenow enzyme, then cloned at the SmaI site of pCOR113. A recombinant plasmid pAN1 was screened by colony *in situ* hybridization (Fig. 6A) with α-^{32}P labelled 300 bp Nos-ter as probe. Identification of pAN1 by restriction digestion was displayed in Fig. 6B. These results confirmed that Nos-ter signal had been cloned downstream of rice actin 1 promoter with correct orientation which was identified by BamHI digestion. The second round of ligation was to in-

sert glnA gene between rice actin 1 promoter and Nos-ter elements. The glnA gene from pGSJ1 was filled by klenow enzyme, then cloned at EcoRV site of pAN1. Positive clones pAGN1 containing glnA gene were selected by using colony *in situ* hybridization with α-^{32}P labelled 1.4 kb glnA fragment as probe (Fig. 7A). The results of restriction digestion for pAGN1 were shown in Fig. 7B. Orientation of glnA insertion was ascertained by PstI digestion, indicating pAGN1 with correct orientation and pAGN2 reverse one. The third round of ligation and transformation were carried out to import an Act1-glnA-Nos-ter fragment from pAGN1 by KpnI-XbaI digestion into KpnI-XbaI cohesive ends of pBIN19. This was the final step for construction of pAGNB92 expression vector. The pAGNB92 plasmid harbored rice actin 1 promoter, glnA gene, Nos-ter and Kanamycin (or G418) selectable marker gene. The restriction digestion of pAGNB92 was given in Fig. 8A. In order to confirm further the actual existence of the three functional elements as mentioned above in pAGNB92, DNA-DNA dot blot hybridization was performed. All these results were shown in Fig. 8B.

2.3 Transformation of Rice Protoplasts with Expression Vectors pGSC35 and pAGNB92, Selection of the Transformants and NPT II Activity Assay in G418 Resistant Calli

2.3.1 Isolation and Transformation of Protoplasts

Fig. 9. showed freshly isolated protoplasts which remained rounded in appearance and highly cytoplasmic. The protoplasts were used to be transformed with expression vectors pGSC35 and pAGNB92 by PEG fusion and electroporation.

2.3.2 Culture of Transformed Protoplasts and Selection of the Transformants

The transformed protoplasts were incubated at 25～27℃ in darkness. First division occurred after 5～8 days of culture as shown in Fig. 10a, b. 12 days later second division occurred in agarose segments with KPR liquid medium (Fig. 10c, d). At the stage of third division or later (about 16 days of culture, Fig. 10e) KR liquid medium was used to replace KPR and mixed with G418 antibiotic at a concentration of 20 μg/ml to select transformants. In this period of selection, compact cell colonies were formed after 22 days of incubation (Fig. 11a). After 30 days of culture G418^r microcalli (Fig. 11b) were transferred to N_{6-1} differentiation solid medium containing 20～25 μg/ml G418 to make further selection. About 6 weeks later, some calli continued to grow rapidly with the size of 0.5～1.0 mm, exhibiting high G418 resistance (Fig. 12a, b).

2.3.3 NPT Ⅱ Activity Assay in G418 Resistant Calli

G418^r calli (60~80 mg per piece) were collected for detection of NPT Ⅱ activity using γ-^{32}P-ATP and Kanamycin as substrates. The results for this assay were shown in Fig. 13a, b. About 37% of G418^r calli tested appeared positive hybridization dots, indicating NPT Ⅱ genes in glnA expression vectors pGSC35 and pAGNB92 had been highly expressed in these calli.

3 DISCUSSION

In this work we have successfully cloned an open reading frame (ORF) glnA gene from *Azospirillum brasilense Sp7* by PCR (Polymerase Chain Reaction) technique. Up to now, it has not been reported that any other GS gene was cloned by PCR technique. Actually it was very rapid and easy for cloning a target gene with PCR technique.

To construct glnA expression vectors we selected two kinds of promoters CaMV 35S and rice actin 1. It was previously reported that rice actin 1 promoter was much more efficient than CaMV 35S for promotion of foreign gene expression in transgenic cereals[9]. Furthermore, in transgenic rice plants the CaMV 35S promoter was not active in all cell types[16]. In contrary, the endogenous actin 1 transcript is abounded in all tissues and development stages of the plant examined[17]. Therefore, the rice actin 1 promoter in glnA expression vector pAGNB92 was believed to hold great superiority in subsequent transgenic rice production.

In this paper we reported the application of ^{32}P isotope in construction of glnA expression vectors and NPT Ⅱ activity assay in the transgenic rice calli. It was very beneficial and simple for α-^{32}P labelled probes to screen the recombinant plasmids, especially to select clones which were bluntly ligated.

NPT Ⅱ enzyme is encoded by neo gene which is one of reporter genes. Positive hybridization dots showed that this reporter gene had been integrated into rice genome. High level of NPT Ⅱ activity from efficient expression of neo gene conferred rice calli strong G418 resistance. These G418^r calli were further induced to regenerate rice plantlets. NPT Ⅱ detection in this study would facilitate the production of glnA-transgenic rice plants in subsequent work.

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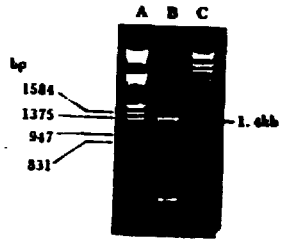

Fig. 1 PCR amplification of glnA from *A. brasilense Sp7*

A: λDNA/R1-H3;

B: PCR products;

C: λDNA/H3.

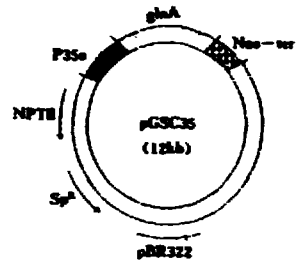

Fig. 3 Structure of pGSC35 carrying glnA gene of *A. brasilense Sp7*

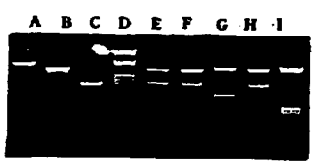

Fig. 2 Identification of pGSJ1 containing glnA from PCR product by restriction digestion

A: pGSJ1/H3: 4.36;

B: pBS/H3: 2.96;

C: recovered PCR product;

D: λDNA/R1-H3;

E: pGSJ1/R1-H3: 2.96, 1.40;

F: pGSJ1/B1: 2.96, 1.40;

G: pGSJ1/S1: 3.26, 0.85, 0.25;

H: pGSJ1/P1: 3.06, 1.30;

I: pGSJ1/Sm1: 3.39, 0.55, 0.42.

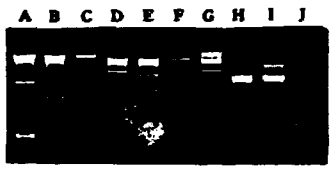

Fig. 4 Identification of pGSC35 by restriction digestion and PCR amplification

A: pGSC35/S1: 10.53, 1.10, 0.27;

B: pGSC35r/S1: 11.09, 0.54, 0.27;

C: pCo24/S1: 10.5;

D: pGSC35/P1: 5.80, 3.40, 1.95, 0.50, 0.25;

E: pGSC35r/P1: 5.80, 3.40, 1.60, 0.85, 0.25;

F: pCo24/P1: 5.80, 3.50, 0.95, 0.25;

G: λDNA/R1-H3;

H: glnA PCR from pGSC35;

I: glnA PCR from pGSC35r;

J: glnA PCR from pCo24.

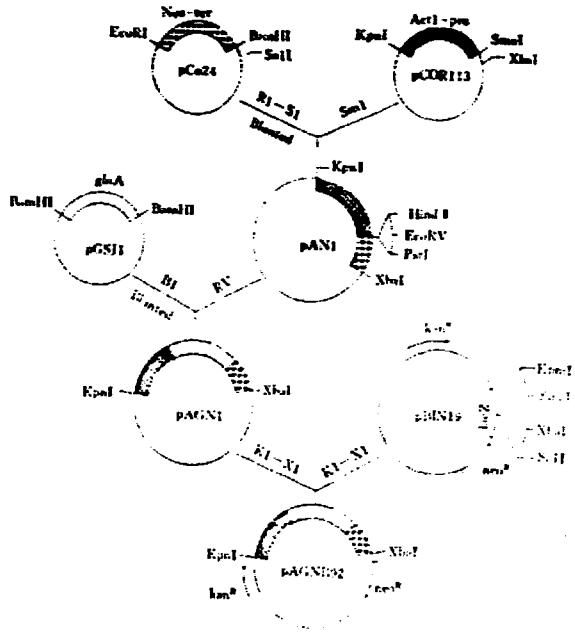

Fig. 5 Construction of glnA expression vector pAGNB02

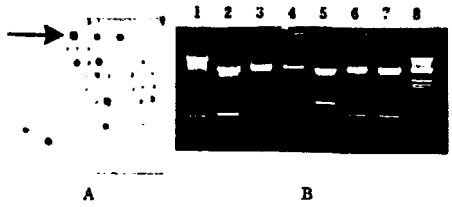

Fig. 6 Colony *in situ* hybridization (A) and identification of pAN1 by restriction digestion (B)

A: Arrow indicated positive control (pCo24).

B: 1. pCo24/R1-S1: 10.2, 0.30;
2. pAN1/H3-X1: 4.40, 0.30;
3. pAN2/H3-X1: 4.40, 0.30;
4. pCOR113/H3-X1: 4.40;
5. pAN1/B1: 4.07, 0.58, 0.05;
6. pAN2/B1: 4.07, 0.38, 0.25;
7. pCOR113/B1: 4.07, 0.33;
8. λDNA/R1-H3.

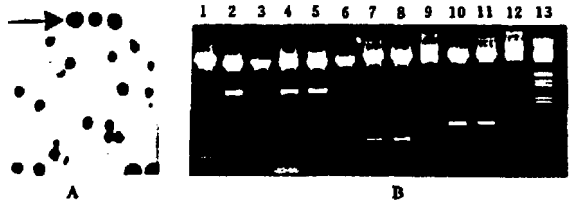

Fig. 7 Colony *in situ* hybridization (A) and identification of pAGN1 by restriction digestion (B)

A: Arrow indicated positive control (pGSJ1).

B: 1. pAGN1/P1: 5.98, 0.12;
2. pAGN2/P1: 4.80, 1.30;
3. pAN1/P1: 4.7;
4. pAGN1/H3-P1: 4.68, 1.30, 0.12;
5. pAGN2/H3-P1: 4.68, 1.30, 0.12;
6. pAN1/H3-P1: 4.7;
7. pAGN1/S1: 5.83, 0.27;
8. pAGN2/S1: 5.83, 0.27;
9. pAN1/S1: No S1 site;
10. pAGN1/Sm1: 5.65, 0.45;
11. pAGN2/Sm1: 5.65, 0.45;
12. pAN1/Sm1: No Sm1 site;
13. λDNA/R1-H3.

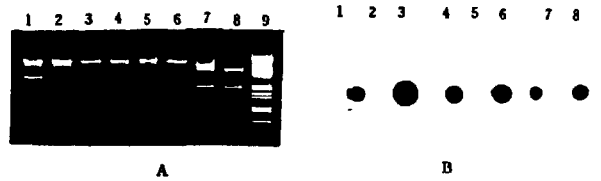

Fig. 8 Identification of pAGNB92 by restriction digestion (A) and Dot blot hybridization (B)

A: 1. pAGNB92/K1-X1: 10.0, 3.10;

2. pBIN19/K1-X1: 10.0;

3. pAGNB92/S1: 12.0, 0.86, 0.24;

4. pBIN19/S1: 10.0;

5. pAGNB92/Sm1: 12.65, 0.45;

6. pBIN19/Sm1: 10.0;

7. pAGNB92/P1: 6.70, 4.00, 2.00, 0.40;

8. pBIN19/P1: 4.00, 4.00, 2.00;

9. DNA/R1-H3.

B: 1, 4, 7=pAGNB92;

Negative control. 2, 5=pBIN19, 8=pBS;

Positive control: 3=pCOR113, 6=pGSJ1, 9=pCo24;

Probes: 1~3=rice actin 1 promoter (1.4kb K1-X1 fragment from pCOR113);

4~6=glnA gene (1.4kb B1 fragment from pGSJ1);

7~9=Nos-ter (300bp R1-S1 fragment from pCo24).

Fig. 9 Freshly isolated rice (var. T986) protoplasts

Scale: 25 μm

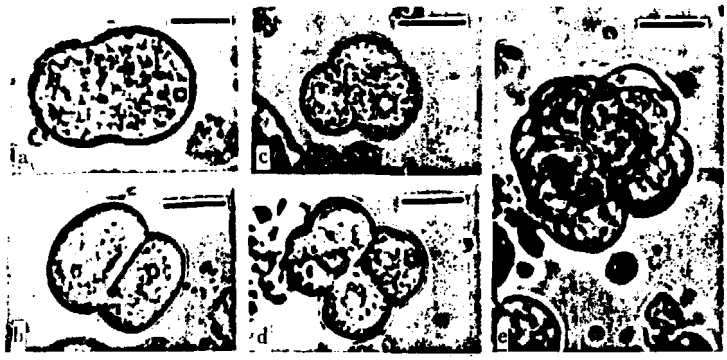

Fig. 10 Culture stages of transformed rice (cv. T986) cells in KPR medium with 0.6% agarose

a: initiation of the first division;

b: the first division;

c, d: the second division;

e: multiple division;

Scale: 50 μm.

Fig. 11 G418' colony development

a: Compact cell colony;

Scale: 200 μm;

b: G418' microcalli.

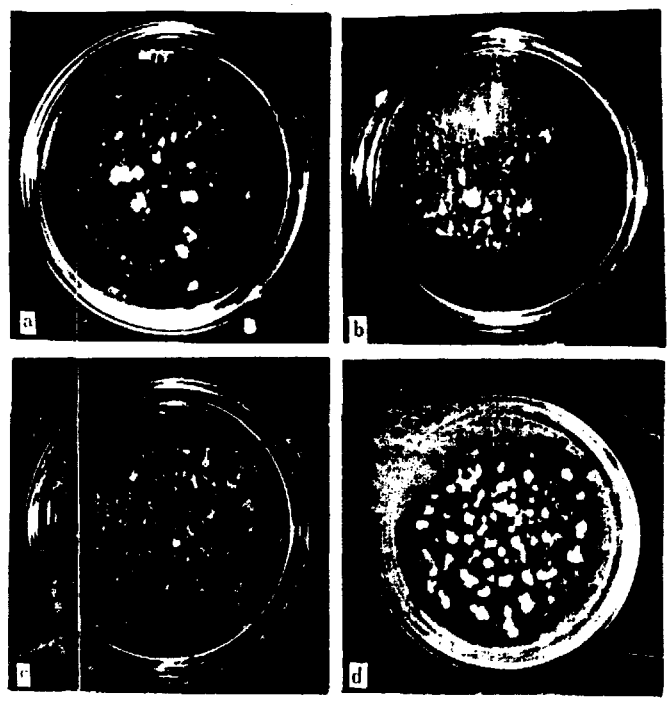

Fig. 12 Selection of G418^r rice transformants on N_6 differentiation medium

a: pGSC35 transformed calli selected with 20 μg/ml G418;
b: pAGNB92 transformed calli selected with 25 μg/ml G418;
c: Negative control with 25 μg/ml G418;
d: Positive control without G418 antibiotic.

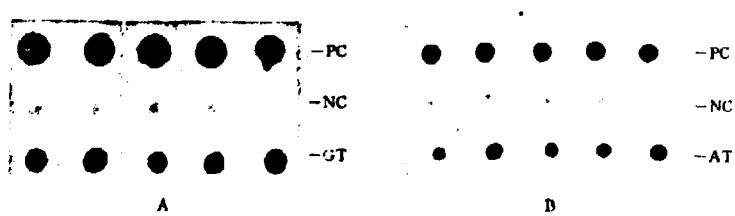

Fig. 13 NPT Ⅱ activity assay in G418^r rice calli by NPT Ⅱ dot hybridization

A: pGSC35 transformation; B: pAGNB92 transformation.
PC (Positive Control), Bacterium (with plasmid pBIN19) extracts containing NPT Ⅱ enzyme.
NC (Negative Control), Untransformed calli.
GT, pGSC35 transformed calli.
AT, pAGNB92 transformed calli; Indiacted numbers mean different callus pieces.

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